The Thesis Committee for Bryan Barlow McClellan
Certifies that this is the approved version of the following Thesis:

The Modulation of the Fatty Acid Synthase Enzyme in Obesity-Induced Breast Cancer

APPROVED BY
SUPERVISING COMMITTEE:

_____

Linda deGraffenried, Supervisor

_____

Christopher A. Jolly

The Modulation of the Fatty Acid Synthase Enzyme in Obesity-Induced Breast Cancer

by

Bryan Barlow McClellan

Thesis

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Master of Science in Nutritional Sciences

The University of Texas at Austin

August 2020

Dedication

I would like to dedicate this page to my family, especially to my mother and father. Their endless love and support are the sole reason I have been able to embark on this incredible journey. As for my siblings, I would like to especially thank my sister for being an excellent role model and an exemplar teacher. I would also like to dedicate this page to my girlfriend, Sarah Ruth Andrews. Her continued support and love has truly bolstered me through this entire endeavor, and for that; I could not be more grateful. Finally, and most importantly, I would like to dedicate this to my late grandmother, Kay Bryan, who passed away from breast cancer. She is what gives me the drive every day keep pushing to find answers to this malicious disease in which so many wonderful people have been taken victim.

Acknowledgments

I would like to thank Dr. Linda deGraffenried for giving me a chance to prove to myself in a translational cancer lab, especially coming from a background not associated with molecular biology. I am forever indebted to you for all that you have done for me, including opening the door to a career path that I never thought was possible. I would also like to thank Dr. DeAngulo for having patience with me as she taught me how to perform immunofluorescence and proper cell culture technique. I will always be appreciative of the time that you took out of your schedule to teach me techniques that are so necessary for any basic and translational research setting. Finally, I would like to thank the members of the deGraffenried lab for all their support. I would have been completely lost if it were not for Brittany Harlow and all her support in cell culturing and other lab techniques. I cannot express my gratitude enough for every person involved in the lab that has enabled me to complete this project.

The Modulation of the Fatty Acid Synthase Enzyme in Obesity-Induced Breast Cancer

Bryan Barlow McClellan, M.S.N.S.

The University of Texas at Austin, 2020

Supervisor: Linda deGraffenried

Obesity is currently an epidemic and confers a more aggressive and often treatment-resistant phenotype. There have been proposed mechanisms of obesity-induced breast cancer progression, however, it is still a continually active area of research with many more questions to be answered. The fatty acid synthase enzyme (FASN) has been an "Achilles Heel" for cancer, considering the large number of tumors that overexpress the enzyme. Inhibitors targeting FASN have been an active area of preclinical research over the past decade, with one, TVB-2640, that is currently in clinical trials. We previously recorded an upregulation in FASN when breast cancer cells were exposed to obese sera compared to breast cancer cells exposed to lean sera, highlighting FASN expression as a possible mechanism behind obesity-induced breast cancer progression. We chose to investigate the role of the Insulin-like growth factor receptor (IGF-1R), which is also upregulated in many aggressive and resistant cancers, as a target of FASN. We hypothesis that the FASN is inducing breast cancer aggression through the increased localization and activation of the IGF-1R to the membrane. To visualize if the IGF-1R was localized as a target of FASN upregulation, we used the FASN inhibitor, TVB-3166. MCF-7, luminal A, and MD-MB-231, triple-negative, cells were treated with obese sera, insulin-like growth factor -1 (IGF-1), or lean sera. Cells

were then either treated with the FASN inhibitor, TVB -3166 or the vehicle control (DMSO) for 72 hours and visualized for localization with immunofluorescence. Upon FASN inhibition treatment, the localization of the IGF-1R the membrane was abrogated compared to the control. These results highlight a potential mechanism in which IGF-1 induced localization of the IGF-1R is mediated through FASN. In summary, FASN highlights a potential therapeutic target in obesity-induced breast cancer with our lab previously demonstrating an accentuated FASN expression in response to obese sera exposure. Also, when FASN is targeted using the FASN inhibitor, TVB-3166, there is a consequential decrease in the IGF-1R localization in response to obese sera and IGF-1, Thus, FASN is a potential target in obesity-induced breast cancer and could participate in tumor progression through IGF-1R localization.

Table of Contents

Table of Figures

Chapter 1: Introduction

1.1 Breast Cancer Epidemiology and Classification

EPIDEMIOLOGY

With the sobering epidemiological data, resulting in 41,760 deaths per year, breast cancer has become an unfortunate prevalence (1, 2). Due to its heterogeneity, breast cancer is not simply one cancer, but a multitude of differing subtypes, each with unique responses to therapies (1, 3, 4). To aid in the identification and treatment of the breast carcinomas, various histological and molecular subclassifications have been established. Moreover, various risk factors can lead to cancer such as those found in the study conducted by Engmann et al. (5), that analyzed nearly 203,000 women for common risk factors associated with their cancer (5). There was an astonishing 44 % of breast cancer estimated to attributed to risk factors such as breast density, family history and body mass index (BMI) in both pre and postmenopausal women (5). Other established risk factors that can drive these epigenetic and genetic alterations that lead to cancer include alcohol intake, diet, weight, hormone use and external environmental exposures (6).

HISTOLOGICAL & MOLECULAR CLASSIFICATION OF BREAST CANCER

The mammary tissue is a heterogeneous mix of epithelial and mesenchymal cells that include adipocytes, fibroblasts, endothelial cells, and immune cells (1, 7). The two main cell types within a mature mammary gland include luminal and basal myoepithelial cells. These two cells can undergo further differentiation into ductal cells or milk-producing cells (7). Previous genetic and molecular profiling has provided the identification of

subtypes of breast cancer, which can aid in proper treatment based on certain characteristics. For instance, the presence

of the hormone receptor, estrogen receptor (ER), is used to classify subtypes and aid in targeted treatment (1, 8). Also, the human epidermal growth factor receptor-2 (HER-2), is used to classify subtypes of carcinomas (1). These can be used in combination with other molecular markers such as progesterone receptor (PgR) and the ki67 marker of proliferation (1, 8). Based on the expression of these markers or lack thereof, subclassifications have been named based on the origin of the lesion and expression of various receptors and proteins. The molecular subtypes of breast cancer include: Luminal A (estrogen receptor-positive), luminal B (estrogen receptor-positive and either HER2 positive or negative), HER2 enriched (expressed HER2), basal-like (estrogen and progesterone receptor negative) and claudin- low or mesenchymal- like (1, 8-10).

1.2 Obesity and Breast Cancer

EPIDEMIOLOGY

Obesity is currently affecting 30 % of the United States (11). In addition to diabetes and cardiovascular disease, obesity has been associated with a higher risk of carcinomas of the esophagus, gastric, thyroid, pancreas, colorectal, endometrium, prostate, gallbladder, and breast (12). The risks for premenopausal women and breast cancer as well as triple-negative breast cancer has mixed results from various studies showing both positive and inverse correlations with breast cancer risk and BMI (11). Moreover, obese, postmenopausal women have a 30 % higher chance of developing breast cancer and are positively associated with each 5 kg/m^2 unit of BMI increase (11). In addition to greater risks associated with obesity, there is a shorter time to disease

recurrence as well as greater mortality in both premenopausal and postmenopausal women with breast cancer (11). The American cancer society cancer prevention study followed almost 500,000 women from 1982 until 1998 and found a significant ($p<.001$) positive association between BMI and breast cancer mortality (11). While there are many ways in which an obese environment can lead to tumorigenesis such as increased leptin, reduced adiponectin, increased adipose tissue macrophage (ATM) infiltration, increased estrogen production, and vascular endothelial growth factor (VEGF) release (13-19) **Figure 1.** ; the primary focus will be on the deleterious effects of hyperinsulinemia and insulin-like growth factors signaling.

OBESITY INDUCED INSULIN RESISTANCE

Obesity induces enhanced intracellular oncogenic signaling through creating an extracellular environment in which mitogenic stimuli are prevalent. One method in which adipocytes can contribute to excess growth stimuli is through the acquisition of insulin-resistant adipocytes (14). Insulin resistance is defined as a reduced response to insulin-stimulated glucose uptake in the liver, muscle and adipose tissue and is hypothesized to be a result of chronic systemic low-grade inflammation commonly seen in obesity (20, 21). In obesity, insulin resistance is ultimately obtained through a defect in insulin receptor activity through mediators such as c-Jun NH2-terminal kinase (JNK 1 and 2), inhibitor of kappa β kinase (IKK), extracellular related kinase 1 & 2, (ERK 1/2), mitogen-activated protein kinase (MAPK), and insulin receptor substrate (IRS) 1 & 2 (20-22). Moreover, insulin resistance signaling mediators in obesity are hypothesized to be downstream effectors from various metabolites and signals which include increased free fatty acids (FFA) and long-chain saturated fatty acids from both diet and endogenous

sources, diacylglycerols (DAGs), reactive oxygen species (ROS), endoplasmic reticulum (ER) stress, tumor necrosis factor-α (TNF-α), and glucose (21).

INSULIN, IGF-1 & CANCER PROGRESSION

Insulin resistance has been associated with an increased risk of breast, colorectal, liver, and pancreatic cancer (15, 16, 23, 24). Moreover, the insulin receptor (INSR), Insulin-like growth factor receptor (IGF-1R), and the downstream adapter protein insulin receptor substrate-1 (IRS-1) have been associated with decreased survival and treatment resistance in breast cancers (23, 24). There are varied mechanisms in which insulin resistance is associated with an increased cancer risk and worse prognosis, including hyperinsulinemia, increased bioactive IGF-1, circulating sex hormones, and ROS due to hyperglycemia (21, 23, 24). Both insulin and insulin-like growth factors (IGFs) result in similar downstream signals for increased cellular survival, proliferation, and differentiation (16, 21, 23, 24). In muscle and liver, the insulin receptor is primarily responsible for the translocation of the GLUT 4 receptor to the plasma membrane for glucose influx in response to an increase in plasma glucose and insulin (21, 24). However, there are isoforms of insulin receptors that respond to both insulin-like growth factor II (IGF-II) and insulin that result in signaling for proliferation and growth in cells (21, 24, 25). The INSR and IGF-1R receptor also can hybridize as one dimeric structure to respond to insulin and IGF-1 (23, 24). Thus, obesity can mediate tumorigenic effects through both insulin and insulin-like growth factor binding for subsequent oncogenic signaling.

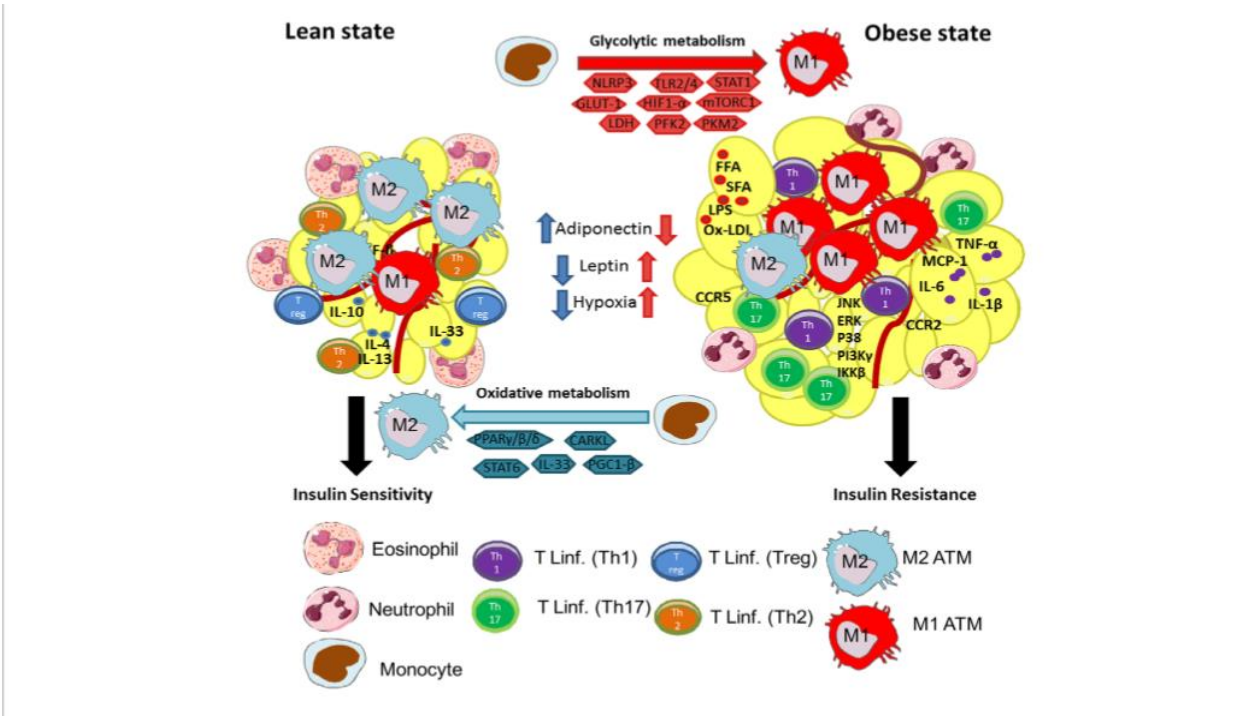

Figure 1. Obese Adipose Tissue

The lean adipose tissue, depicted on the left consists of a majority of M2 adipose tissue macrophages (ATM). These are associated with secreted anti-inflammatory cytokines such as IL-10, IL-4, IL-13, IL-33. Also, these adipocytes secrete signaling molecules such as peroxisome proliferative activated receptor (PPAR) and STAT 6 which favor oxidative metabolism. The lean adipose tissue also have Th2 T cells, eosinophils, and regulatory T cells (Tregs). Conversely obese adipose tissue favors glycolytic metabolism by activation of glycolytic metabolism regulators such as lactate dehydrogenase (LDH), STAT 1, and hypoxia-inducible factor 1 – α (HIF-α). The M1 macrophages secrete cytokines such as TNF-α, IL-6, IL-1β, and other inflammatory molecules. The obese adipose tissue also contains more Th17 and Th1 lymphocytes as well as fewer eosinophils and more neutrophils. This image was obtained from Castoldi, Naffah De Souza (20).

1.3 The IGF-1 System

BASIC STRUCTURE AND FUNCTION OF INSR & IGF-1R

The IGF signaling cascaded is an important and complex sequence of signals that are primarily responsible for cell proliferation and survival (26). There have been many studies showing the essentiality of IGF-1R in tumor development as well as both an overexpression of the IGF-1R/INSR hybrid and the downstream adapter protein, IRS-1 in tumors (26-30). The IGF signaling pathway is initiated primarily by three ligands, insulin, IGF-1, and IGF-2 (26). However, there are a few more members IGF family such as the ligand antimicrobial peptide LL-37, the orphan insulin-related-receptor-receptor (IRR), the insulin-like growth factor-1 – insulin hybrid receptor (26). The main receptor tyrosine kinases involved are the insulin receptor (INSR), insulin-like growth factor-1 receptor (IGF-1R), and insulin-like growth factor-2 receptor (IGF-2R). The insulin receptor itself has two spliced isoforms: INSRA and INSRB (27). The INSRA isoform contains exon 11 and responds to mainly insulin stimulation, while the INSRB form lacks exon 11, is activated by IGF-II, and is associated with various cancers (27). Both the INSR and IGF-1R share up to 84% identity in the tyrosine intracellular domains (26). Moreover, there is a complexity of signaling networks in which the IGF-1R “crosstalk’s” with other RTKs such as the epidermal growth factor receptor (EGFR), G-protein coupled receptor (GPCR), and the INSR by transactivating the INSR tyrosine kinase domains (26). The receptors are composed of both an extracellular glycosylated α subunit and an intracellular β subunit (26). The IGF-1R begins as Pro-IGF-1R or a preformed αβ chain that is activated by glycosylation and proteolysis (26) that eventually transitions into a mature form results in two identical α and β chains **Figure**

2. The majority of the receptor is composed of the β chain, which consist of an extracellular domain, transmembrane domain, juxtamembrane domain, and an intracellular tyrosine kinase domain (26, 27). These subunits, such as the α subunit consist of 710 amino acids separated by a cysteine-rich domain, which is the primary ligand-binding region for both IGF-1R and INSR (26). The beta subunit of the IGF-1R contains a 196 amino acid extracellular domain, a 926 amino acid transmembrane domain, and an intracellular domain that contains three different regions which include a juxtamembrane domain, a tyrosine kinase domain, and a carboxy-end terminal tail (26). The juxtamembrane contains residues necessary for IGF-1R internalization, while the tyrosine kinase domain contains three essential tyrosine residues that are necessary for activation of the receptor (26, 27). The structure and conformation of the activation loop in the tyrosine kinase domain of the receptor prevents any ATP binding and acts as an autoinhibitory mechanism (26, 27). Due to the location of the activation loop within the domain, the tyrosine residues located near the loop are extremely important for the receptor activation.

IGF-1R SIGNALING & REGULATION

Ligand binding by IGF-1/2 causes the tyrosine 1135 to become trans- phosphorylated by adjacent dimers in the activation loop of the IGF-1R (26, 27). This phosphorylating event continues down the tyrosine kinase domain where tyrosine autophosphorylation attracts certain adapter proteins (26, 27). In particular, tyrosine 950 in the juxtamembrane domain seems to be the major tyrosine residue in which Shc and IRS adapter proteins are attracted (26). The tyrosine phosphorylating events provide signals for proteins that contain (src homology 2) SH2 and phosphotyrosine binding (PTB)

domains (26, 27). One of the fastest arriving adapter proteins is the insulin receptor substrate (IRS), which contains a PTB domain (26). The IRS proteins also have c-terminus end that interacts with SH2 domain-containing proteins such as the p85 catalytic subunit of Phosphoinositol- 3 kinase (PI3K) and growth factor receptor-bound protein -2 (Grb2) (26, 27). Another adapter protein that responds to tyrosine phosphorylation of IGF-1R is the Shc (SH2 containing protein) class of proteins (26, 27). Shc and IRS family of proteins all consist of PTB domains at the N- terminus along with an SH2 domain at the C terminus and ultimately lead to recruitment and subsequent signaling molecules that lead to tumorigenesis (26).

PI3K-AKT AXIS

One mechanism by which IGF-1R leads to tumorigenesis and survival is through the Phosphatidylinositol- 3-kinase (PI3K) pathway **Figure 3**. The PI3K genes, PI3KCA, PI3KCB, and PI3KD, are often mutated in many cancers including breast cancer (31). The regulatory subunit, p85, and the catalytic subunit, p110, make up the PI3K complex (26, 32). PI3K's main function is the production phosphatidylinositol (3,4,5) trisphosphate (PIP3) from and phosphatidylinositol (4,5) biphosphate (PIP2) that is formed through the phosphorylation of PIP2 at the 3 ' OH position (26, 31). Phosphorylated tyrosines recruit adapter proteins such as IRS and Shc that bind to downstream effector molecules (26, 27). IRS-1, for example, can bind to a subunit of PI3K, p85 (26). The activation p110 from p85 results from an alleviation of inhibition in the catalytic domain of p110 (26). PI3K's phospholipid product PIP3 can recruit (pleckstrin homology) PH domain-containing proteins such as phosphoinositide-dependent kinase (PDK) and protein kinase B/Akt (26, 27). Upon recruitment to the

membrane via PIP3, PDK can phosphorylate Akt at threonine 308 and allow it to signal to downstream targets that are involved in survival and proliferation (26, 27). Once Akt is membrane-bound and activated by either serine 473 or threonine 308 phosphorylation, it can target pro-survival and growth proteins (26, 27). Akt carries out its pro-survival signaling by inhibiting apoptotic proteins such as Bad, BAX, and caspase 9 (26). Also, it inhibits glycogen synthase kinase-3 β (GSK-3β) and forkhead box transcription factors (FOXO) (26, 31). Akt activates a major regulator in protein synthesis and G0 to G1 cell cycle transition, mechanistic target of rapamycin complex 1 (mTORC1) (26, 31). Activation of mTORC1 by Akt is mediated through Akt phosphorylation and inhibition of the tuberous sclerosis complex 2 (TSC2) (31). Both TSC 1 & 2 have been described as tumor suppressors due to their inhibitory role in mTORC1 activation through their properties as GTPase activating proteins (GAPs) (31). For mTORC1 to be activated, there must be ample bound GTP to Ras homolog enriched in brain (Rheb), which is a small GTPase that controls the activation of mTORC1 (33). Thus, TSC 1 & 2 inhibit mTORC1 activation through the hydrolysis of GTP, resulting in Rheb inhibition (33). Ultimately, mTORC1 leads to cell cycle progression, lipogenic metabolic programming, protein translation, and survival resulting in tumor progression.

MAP KINASE CASCADE

A second way in which IGF-1R can facilitate in carcinogenesis is through the mitogen-activated protein kinase (MAPK) signaling cascade **Figure 3** (26). Once phosphorylated, intracellular tyrosine residues can attract src homology containing (Shc) adapter proteins such as Grb-2 (26, 27, 31). Grb-2 interacts with the

phosphorylated tyrosine's through its SH2 domains and subsequent interaction with effector molecules can be made possible by its SH3 domains which interact with proline-rich motifs present in effector proteins such as sons of sevenless (SOS) (26, 27, 31). SOS is a guanine nucleotide exchange factor that aids in the release of GDP from small GTPases such as Ras (26, 27). Once GDP is release from Ras, GTP can bind and result in an activated conformation in the protein (26, 27). A product of Ras is the serine/threonine kinase, Raf (26, 27). Upon activation, Raf can participate in a phosphorylating cascade that travels down until it reaches the nucleus where various transcription factors facilitate cell cycle progression and cell migration (26, 27). The phosphorylation cascade begins with Raf's phosphorylation of MAPK (MEK), which phosphorylate the MAPK sub-proteins extracellular related kinase (ERK) 1 and 2 (26, 31). Both ERK 1 & 2 can localize to the nucleus or target cytosolic proteins (26, 31). The target proteins of ERK 1&2 are involved in cell-cycle progression and ultimately proliferation as well as cell migration through spindle formation (26, 31). Some specific downstream targets of MAPK and ERK that relate to tumor progression are cyclin D1, along with its respected cyclin-dependent kinase, which results in the phosphorylation of the tumor suppressor protein retinoblastoma (pRB) (26, 31). Phosphorylation of pRB results in histone acetyl transferase recruitment and E2F activation, which controls G1 to S phase transition that allows cells to proliferate (26, 31).

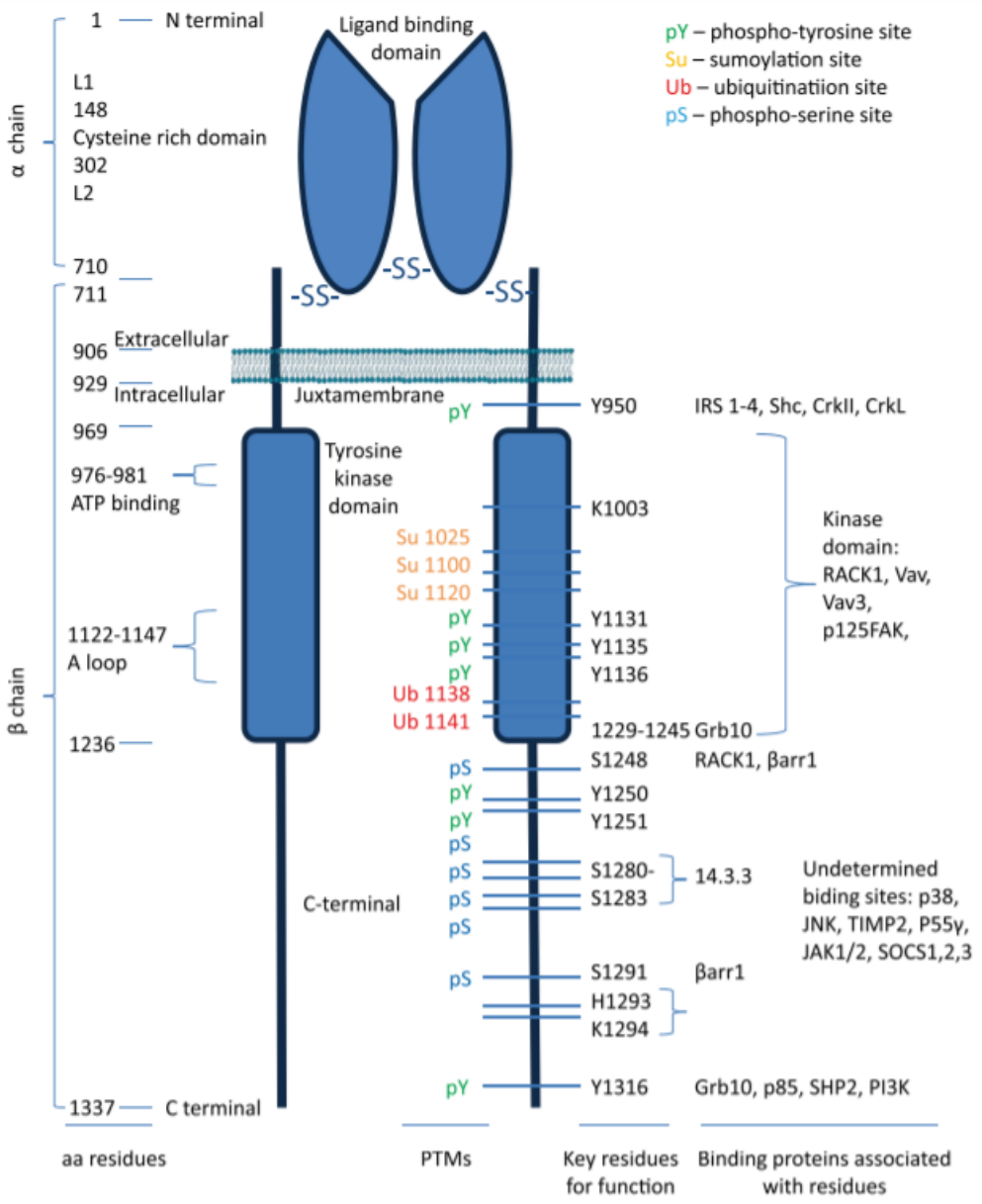

Figure 2. Insulin-like Growth Factor Receptor

Insulin-like growth factor receptor structure. There are four known sites for the post-translational modifications of phosphorylation, sumoylation, and ubiquitination. The N-terminus region is the extracellular portion in the alpha chain region. The alpha chain contains two domains, L1 and L2 separated by a cysteine-rich domain. The two dimers are held together by disulfide bonds. The juxtamembrane domain lies just beneath the

membrane and contains an essential motif for receptor internalization. The tyrosine kinase domain has essential tyrosine residues for phosphorylation Y 1131, Y1135, Y1136. The c- terminal region spans 100 amino acids and plays a regulatory role in the receptor activation/deactivation. Some of the adapter proteins that respond to tyrosine phosphorylation are Grb, p85, SHP2, and PI3K all of which bind to Y1316. Some other adapter proteins such as IRS-1, Shc, CrkII, and CrKL bind to Y950 closer to the cellular membrane. This image was obtained from (26, 31).

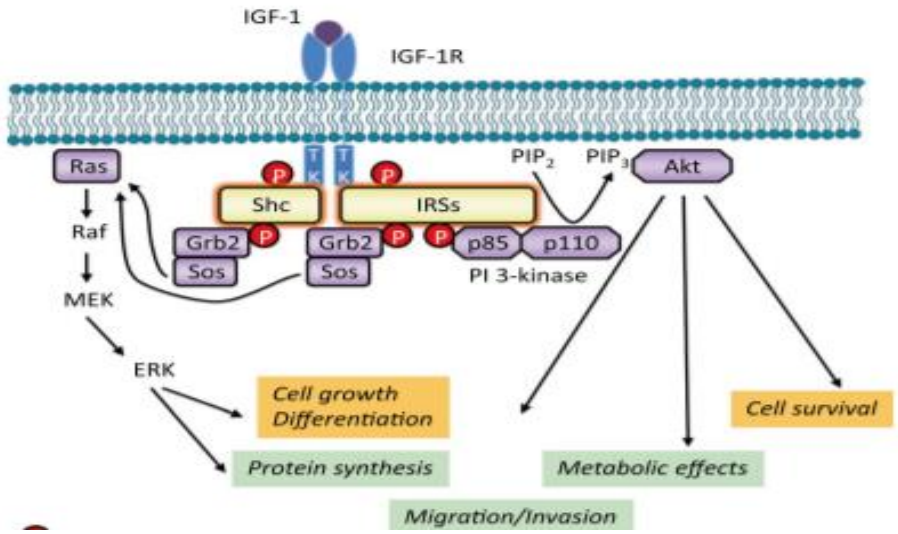

Figure 3. RTK Downstream Signalling

The signaling cascade upon tyrosine kinase autophosphorylation of the IGF-1R. SH2 domain-containing adapter protein responds to phosphorylated tyrosine residues and phosphorylates other adapter proteins like growth factor receptor-bound protein -2 (Grb2) and sons of sevenless (SOS) which can activate downstream mitogenic signaling such as RAS-RAF-MEK- ERK leading to cell proliferation and growth. The insulin receptor substrate (IRS) adapter protein can also bind from its phosphotyrosine binding domain. It can subsequently phosphorylate Grb-2 and the p85 subunit of PI3

kinase leading to lipid signaling PIP2 to PIP3 and Akt activation for a variety of pro-survival and proliferation effects.

1.4 Fatty Acid Synthase

BASIC STRUCTURE AND FUNCTION

Fatty acids are an essential part of every living organism. The biosynthesis of fatty acids for membranes aid in cell division and signaling and is an important biological process that is ubiquitously expressed in all living tissues. The primary enzyme responsible for the biosynthesis of lipids or *de novo* lipogenesis (DNL) is the fatty acid synthase enzyme (FASN) (34-36). FASN is classified as a multifunctional protein and thus is responsible for not only endogenously creating long-chain saturated fatty acids, but also as an energy storage enzyme in which it takes excessive carbon influx and stores it as triglycerides in adipose tissue for subsequent β-oxidation (34, 37, 38). FASN is a homodimeric enzyme in which two ~250 kDa monomers are composed of 7 catalytic enzymes with an acyl carrier protein (ACP) that contains a 4' phosphopantetheine prosthetic group covalently attached to the serine hydroxyl of the ACP (34, 36). The N-terminus contains 3 catalytic domains, while the C-terminus contains 4 (34). FASN is structured in a head to tail fashion, where the three domains in the N-terminus and the four domains in the C-terminus are separated by 600 residues (34). The subunits, ranging from the N-terminus region to the C-terminus tail are β- ketoacyl synthase (KS), acetyl/malonyl transacylase (AT/MT), β-keto acyl reductase (KR), 3-hydroxy acyl ACP dehydratase (DH), Enoyl Reductase (ER), along with two additional Acyl Carrier Protein (ACP), and Thioesterase (TE) groups (34, 36).

De Novo LIPOGENSIS

One of the more important functions of FASN is the production of the 16-carbon saturated fatty acyl chain, palmitic acid. FASN forms palmitic acid via *de novo* lipogenesis (DNL) from the two substrates acetyl coenzyme A (acetyl- CoA) and malonyl coenzyme A (malonyl Co-A), while using nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent (34, 37, 39, 40). Since, DNL occurs outside the mitochondria and in the cytosol, acetyl-CoA must transport out of the mitochondrial membrane in a different form. Excessive amounts of pyruvate fed into the tricarboxylic acid cycle (TCA), leads to the production of high amounts of citrate produced from the combination of oxaloacetate and acetyl-CoA by the enzyme citrate synthase (37, 39). Citrate is subsequently transferred out of the mitochondrial matrix and converted back into acetyl-CoA and oxaloacetate via ATP citrate lyase (ACYL) (37, 41, 42). Acetyl-CoA, in a carboxylation reaction using carboxybiotin to donate a CO_2, is converted to malonyl-CoA from the enzyme Acetyl-CoA carboxylase (ACC) (36, 37). NADPH is available both from the conversion of malate back to pyruvate via the malic acid enzyme as well as from the pentose phosphate pathway (34, 37). Once the conditions are met, the synthesis of palmitic acid can commence. The complex synthesis reaction that leads to the production of palmitic acid was first discovered in Escherichia Coli (E. Coli) as the type 2 fatty acid synthase enzyme (34, 36). The series of reactions add two carbons per cycle and repeats seven times until a final palmitic acid product is formed. The condensation reaction uses two carbons from the acetate in the Acetyl-CoA as a primer, while malonyl-CoA acts as the chain extending donator (34, 36). Using its three carbons, malonyl-CoA donates two carbons to the acetate moiety, releasing the third carbon as CO_2 (34, 36). The entire reaction can be illustrated by the equation:

Acetyl -CoA + 7 malonyl- CoA + 14 NADPH + 14 H^+ → Palmitic Acid + 7 CO_2 + 14 NAD^+ + 6 H_2O

Figure 4. *de novo* Lipogenesis

The FASN enzyme has two main sites for the covalent attachment of acyl carbons. The sites include the thiol on the cysteine of the β-ketoacyl synthase (KS) and the Acyl Carrier Protein (ACP) with the prosthetic 4' phosphopantetheine group (34, 36). The series of condensation and decarboxylative reactions, which are represented in **Figure 4. ,** are carried out by four main reactions: condensation, reduction, dehydration, and a final reduction (34, 36). The first step to the biosynthesis of palmitic acid, termed the substrate loading phase (34, 36), involves the recycling of the CoA thioester group from both the acetyl and malonyl moieties by the acetyl/malonyl transacylase (AT/MT) enzymes (34, 36). The acetate moiety covalently attaches to the thiol of the cysteine on the KS domain, while the malonate moiety is attached to the thiol of the 4'phosphopantetheine (36). The next set of reactions are a part of the chain elongation phase. In the initial step, β-ketoacyl synthase (KS) catalyzes the condensation of the two substrates to ultimately form acetoacetyl-ACP and resulting in a release of CO_2 (34). Next, in an NADPH dependent reaction, B-ketoacyl reductase (KR) reduces acetoacetyl-ACP to the β-hydroxy butyryl-ACP (34). B-hydroxy butyryl-ACP undergoes a dehydration reaction with the aid of the β-hydroxy acyl dehydrase enzyme (34). The β-hydroxy butyryl -ACP group loses water, ultimately forming a double carbon bond and subsequently forms butenyl-ACP (34). The following reaction is another NADPH dependent reduction reaction carried out by the enoyl reductase (ER) enzyme, ultimately resulting in the formation of butyryl-ACP (34). The KS domain subsequently transfers the 4- carbon butyryl group on the ACP to its on cysteine thiol residue, leading

to ACP being able to accept another transfer of carbons from malonate moiety for further chain elongation (34). This malonate loading and sequential transfer of its two carbons to the acetyl group continues until palmitoyl-ACP is formed. With the use of the water that was previously released by the dehydration reaction, the thioesterase enzyme cleaves and releases the S-ACP group from palmitoyl-ACP resulting in a palmitate product.

1.5 FASN: Recent Advances and Clinical Implications

FASN is not highly expressed in many healthy tissues with exception to cycling endometrium, lactating breast, liver, and adipose tissue (37, 39, 40, 42). The saturated fatty acid products of FASN, palmitate (C16:0), myristate (C14:0), and stearate are essential for membrane fluidity as well as post-translational modifications of proteins and subsequent membrane localization via palmitoylation and myristylation (34, 39). Also, the long-chain fatty acids stearate and palmitate can be used for further acyl chain elongation to produce very-long-chain fatty acids, which are used as membrane lipids such as sphingolipids, ceramides, and glycolipids all of which are essential for normal cell division and growth (34). Numerous studies have shown the upregulation of FASN in cancers including ones conducted by Cui et al., (43) that recorded an increase in FASN by immunohistochemistry analysis in 50 patients with breast cancer without an increase in neighboring non-tumorigenic tissue in the breast (43). In the same study, there was an increase in apoptosis in both triple-negative and ER-positive breast cancer cell lines, and apoptosis was accompanied by a concomitant increase in NADPH, highlighting a new potential mechanism of FASN induced cancer progression (43). FASN has been targeted for therapy in cancer for the past twenty years in the

preclinical setting, however, early FASN inhibitors resulted in off-target toxicities and side effects. A FASN inhibitor currently in clinical trials, TVB-2640, has shown promise with very good patient tolerance (44-46). Also, another FASN inhibitor is emerging as a promising treatment, Fasnall, targets the keto-acyl reductase domain in FASN (47). Moreover, both the FASN inhibitors Fasnall and C75 attenuated growth and proliferation in HER2 positive cell lines along with ER-positive cell lines expressing HER2 (47, 48). Moreover, the effect on FASN inhibition on endocrine dependent breast cancer has also been demonstrated (49). Apoptosis in breast cancer cells in response to FASN inhibition has been well established previously, however, precise mechanisms have not been elucidated and hold great importance for increasing efficacy of FASN inhibitors as well as highlight potential combinatorial treatment options. There have been studies elucidating the connection to the FASN product palmitate and receptor tyrosine kinase function, such as the localization of the c-MET receptor (50). Palmitate, FASN's product, undergoes S-palmitoylation by a family of 23 known zDHHC palmitoyl acyltransferase enzymes resulting in an increased affinity for membranes, more efficient folding and stability, increased protein to protein interactions (51). Moreover, a study by Kristin Runkle et al., (52) illustrated that the inhibition of zDHHC 20 palmitoyl transferase enzymes increases signaling in EGFR (52). Conversely, inhibition of FASN using orlistat, resulted in a ubiquitination and attenuation of signaling of mutant EGFR in non-small cell lung cancer (NSLC), further highlighting the complexity and need for studying the connection between FASN and receptor tyrosine kinase signaling in cancer (53). Moreover, studies have illustrated that mutating essential cysteine residues for palmitoylation in membrane proteins abrogates IGF-1R localization (54) to the

membrane as well as FASN inhibition resulting in a decreased total IGF-1R expression (55). Thus, FASN has multiple approaches in contributing to tumor progression, but the research is lacking in the mechanisms behind the connection to FASN modulation and RTK signalling.

Chapter 2: Insulin-like Growth Factor Receptor as a Target of FASN Modulation in Obesity Induced Breast Cancer

2.1 Abstract

INTRODUCTION Breast cancer is among the highest prevalence of cancer-related death in post-menopausal women with increases commensurate with body mass index (BMI). The long-chain saturated fatty acid products of FASN, such as palmitate, have been known to post-translationally modify proteins and receptors and consequently result in an increased affinity and localization to the plasma membrane. Currently, the localization of the receptor tyrosine kinase, Insulin-like Growth Factor Receptor (IGF-1R), in response to increased expression of fatty acid synthase is being investigated as a mechanism of obesity-induced breast cancer progression. **METHODS** To visualize the localization of the IGF-1R in response to FASN expression, MCF-7 cells were treated with a FASN inhibitor and either obese or non-obese sera and subjected to immunofluorescence. **RESULTS** MCF-7 cells treated with TVB-3266 for 24 hours resulted in an abrogated membrane localization of IGF-1R. **CONCLUSION** This study provides mechanistic insight into how the expression of FASN could be contributing to obesity-induced breast cancer progression through the localization of the IGF-1R. FASN inhibition could be a promising therapy for obesity-induced breast cancer.

2.2 Introduction

Obesity, is a rapidly rising epidemic (56) with a prevalence of up to 34% in the United States (57). Breast cancer is among the highest prevalence of cancer-related death in post-menopausal women with increases commensurate with BMI (18, 58). Previous studies, including in our lab, have demonstrated an augmented aggression in breast cancer when exposed to obese conditions (18, 59, 60). The mechanism behind the more aggressive phenotype of breast cancer in obese individuals has become an active area of research, however, the precise modes of action remain enigmatic. The obese phenotypic characterization is associated with an increased energy storage that results in enlarged adipocytes that can become hypoxic and ultimately necrotic resulting in the recruitment of macrophages and the formation of “crown-like structure” hallmark of obese adipocytes(61, 62). Also, adipocytes can lose their ability to accommodate excess lipids resulting in increased adipocyte lipolysis and free fatty acid (FFA) release resulting in insulin resistance (12, 13, 62). There are multiple proposed mechanisms in which obesity can lead to enhanced tumorigenesis, such as elevated lipids and lipid signaling, inflammation, adipokines, and insulin signaling (62). Both our lab and others have recorded an increased FASN expression in response to obese sera in both adipocytes and cancer cells such as breast, endometrial, and prostate (37, 39, 62). FASN in normal tissues, with exception to lactating breast, cycling endometrium, and adipocytes is not highly expressed due to exogenous dietary fatty acids (FA) being the predominant source (37, 39). The lipogenic phenotype of cancer, in which lipids are endogenously synthesized from excess intake of glucose, has been well established and thus has been a popular “Achilles heel” for cancer metabolism targeted therapies

(37, 42, 63). FASN, in an NADPH dependent manner, acts to endogenously biosynthesize long-chain fatty acids, store FA in adipocytes for subsequent ß-oxidation, and produce phospholipids for membranes (37, 39). The overexpression of FASN in tumor types as well as adipocytes has made FASN a possible target for therapy in obesity-induced cancer. Some previous FASN targeted inhibitors included, the Curelinin, Orlistat, epigallocatechin-3gallate (ECGC), and other flavonoids (41). However, early FASN inhibitors were limited in their use as a therapeutic agent for cancer patients because they induced cachexia,(64) which is detrimental to cancer patients undergoing treatment. Newer, more targeted inhibitors toward FASN have shown promise in early clinical studies, and currently the FASN inhibitor, TVB-2640, is in phase II of clinical trials for the treatment of multiple cancers including breast administered as both monotherapy as well as in combination with paclitaxel (46). There are multiple proposed mechanisms of how FASN inhibitors promote cancer cell apoptosis including the reduction of palmitate for post-translational protein modifications and membrane composition, toxic accumulation of the FASN substrate Malonyl-CoA, decreased membrane synthesis, and disruption to lipid rafts. (37, 39-41, 63, 64) . In an interesting study utilizing a novel FASN inhibitor, TVB 3567, and tracking tumor metabolism by isotopically labelled [U-^{13}C] glucose and [^{13}C] C16:0 palmitate; Daniel Benjamin et al. , found that 231-MFP and MCF-7 breast cancer cells not only decreased the *de novo* glucose derived palmitate synthesis but cell viability was also significantly decreased in response to FASN inhibition (41). Receptor tyrosine kinases, such as the Insulin-like growth factor receptor (IGF-1R), are commonly mutated and upregulated in cancers and thus are popular targets for therapeutics (28, 65). Also, a

study by Jang et al., (54), investigated the palmitoylation of membrane proteins and their involvement in the localization of growth receptors to the plasma membrane. Jang et al. (54), found when the membrane protein flotillin-1 palmitoylation was defected using a palmitoylation-deficient cysteine 34 (C34A) mutant, the co-localization of IGF-1R from the endoplasmic reticulum to the plasma membrane was inhibited by up to 40% (54). The downstream oncogenic signaling cascades of IGF-1R, as well as other receptor tyrosine kinases (RTK), involve activation of two major pathways Raf activated kinase/ mitogen-activated protein kinase (RAF/MAPK) as well as the phosphatidylinositol- 3-kinase/protein kinase B (PI3K/AKT) pathways (26). These ultimately lead to downstream signaling such as cellular survival, growth, and differentiation (26).

There have been numerous studies elucidating the effectiveness of FASN inhibitors for the treatment of breast cancer, however, there have been few studies illustrating a connection to FASN expression and IGF-1R function in breast cancer. Thus, this study is investigating FASN as a contributor to breast cancer aggression through the localization of the IGF-1R. The results from this study provide insight into a mechanism by which obesity promotes a worse prognosis in obese breast cancer patients.

2.3 Methods

Antibodies

Rabbit polyclonal antibodies against IGF-1R β (cat#3027S), p-IGF-1R β tyrosine (1150/1151) (cat#3024) were purchased from Cell Signalling Technology (Danvers, MA, USA). For immunofluorescence, FITC conjugated anti-mouse IgG secondary antibody was purchased from Abnova Corporation (cat#PAB4971).

Serum Samples

Serum was collected from postmenopausal women. BMI was calculated and serum was pooled according to the BMI of the patient (non- obese (Non- OB: 18.5-24.9 kg/m^2); obese (OB:≥ 30 kg/m^2)).

Cell Culture

MCF-7 and MDA-MB-231 human breast cancer cell lines were maintained in essential minimum essential medium (EMEM) (GIBCO Life Technologies, Grand Island, USA) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.

Growth Factor and FASN Inhibition Treatment

The FASN inhibitor, TVB-3166, was acquired from Dr. Andrew Brenner. Human recombinant Insulin-like growth factor (IGF-1) was purchased from R&D systems (cat#291-61). MCF-7 and MDA-MB-231 cells were treated either with or without IGF-1 (10nM) for 60 minutes and with TVB-3166 (200 nM) or with the drug vehicle dimethyl sulfoxide DMSO for either 24 or 72 hours and were subsequently subjected to further analysis.

Immunofluorescence

MCF-7 cells were seeded in complete EMEM supplemented with 10% FBS at a density of 10,0000 cells per 8- well chamber slides (Lab-Tek II Chamber Slide System cat#152941). Cells were exposed to 2% non-obese and serum-free media (SFM), 2% Obese + SFM , 2% Obese + DMSO + SFM , and 2% Obese + TVB-3166 + SFM for 24, 48, and 72 hours. Media was removed after respected incubation time and washed with 1x phosphate-buffered saline (PBS). The cells were then fixed in 4% Formaldehyde in 1xPBS followed by three 1xPBS washes. The cells were then blocked in a normal goat

serum (Novex Life Technologies, cat#PCN500) blocking buffer solution. After blocking, the primary antibody IGF-1R β (cat#3027S) was added overnight at 4C followed by the secondary FITC conjugated anti-mouse IgG antibody (Abnova Corporation cat#PAB4971). The chamber was then removed, washed three times in 1xPBS and mounted in Invitrogen prolong gold antifade reagent with DAPI (Thermo Fisher Scientific, cat#P36941). The slides were allowed to dry for 24 hours and then visualized using a LEICA confocal microscope at 63X.

2.4 Results

FASN Expression Results In IGF-1R Localization.

IGF-1R is upregulated in many cancers and is associated with both drug resistance and a poor disease outcome in estrogen receptor-positive breast cancers (28). IGF-1R activation by insulin and IGF-1 results in tyrosine phosphorylation and recruitment of adapter proteins and downstream oncogenic signaling through RAS-RAF- mitogen-activated protein kinase (MAPK) and PI3K-Akt for survival and proliferation (26). Moreover, long-chain fatty acid products of FASN have been previously demonstrated to post-translationally modify and stabilize proteins and receptors for enhanced oncogenic signaling (54, 66). To visualize the role of FASN and the localization of the IGF-1R to the plasma membrane, MCF-7 cells were treated with either 2% non-obese sera, 2% obese sera, 2% obese sera + DMSO, and 2% obese sera + TVB-3166. The slides were fixed and mounted with anti- IGF-1R β (cat#3027) and FITC secondary antibody along mounting fluid with DAPI nuclear stain. Subsequent imaging using a Leica confocal microscope (63X) resulted in an increased localization of IGF-1R in the obese and obese group **(Figure 5 A)**. Treatment with TVB-3166 + obese sera resulted

in a decreased localization **(Figure 5 A&B)**. These results reveal a new potential connection to FASN expression and IGF-1R localization in obesity-induced breast cancer.

2.5 Discussion

While the link between obesity and cancer progression is firmly rooted, the specific roles that obesity plays in accentuating tumorigenesis are still being elucidated. In the present study, we demonstrated a possible link between obesity and increased breast cancer aggression through FASN induced IGF-1R localization. We previously recorded an increase in FASN expression in cancer cells exposed to 2% obese sera compared to cancer cells exposed to 2% non-obese. Also, inhibition of FASN by treatment with TVB-3166 reduced localization of the IGF-1R to the membrane during both IGF-1 and obese sera exposure. Though we did not elucidate a mechanism in which the FASN inhibition attenuates localization of the IGF-1R to the membrane, increased amounts of the FASN product palmitate could be a contributor. Palmitate, through the zDHHC palmitoyl acyltransferase family of enzymes, is known to aid in the affinity of proteins to lipid rafts within the plasma membrane (66). There has not been evidence of direct palmitoylation of IGF-1R, however, studies have shown other proteins with palmitoylation sites cysteine residues that are responsible for the localization of IGF-1R. For example, studies by Jang et al., highlight the imperative role of the flotillin-1 membrane protein and its palmitoylation for the intracellular transport of IGF-1R (54). The palmitoylation of the cysteines on the membrane proteins might explain a possible mechanism in which FASN causes the increased localization of the IGF-1R. Moreover, an increase in *de novo* lipogenesis has been established previously in cancers and breast cancers alike,

but have not been attributed as a link between obesity driven cancer aggression. While many mechanisms are currently being investigated to understand how the correlation occurs, obesity-induced FASN upregulation has been identified as playing a role. Future research efforts should try and identify the effect that palmitate has in localization of the IGF-1R as well as the effects of this localization on subsequent drug sensitivity and cell viability. As our breast cancer patient population becomes increasingly obese, it is of utmost need to find novel therapeutics to combat the obesity-induced cancer growth. Based on the results, FASN inhibition appears to show promise as a nontoxic, novel therapeutic for the treatment of obesity-induced breast cancer.

Figure 5 (A-B): FASN Inhibition Abrogates IGF-1R Membrane Localization

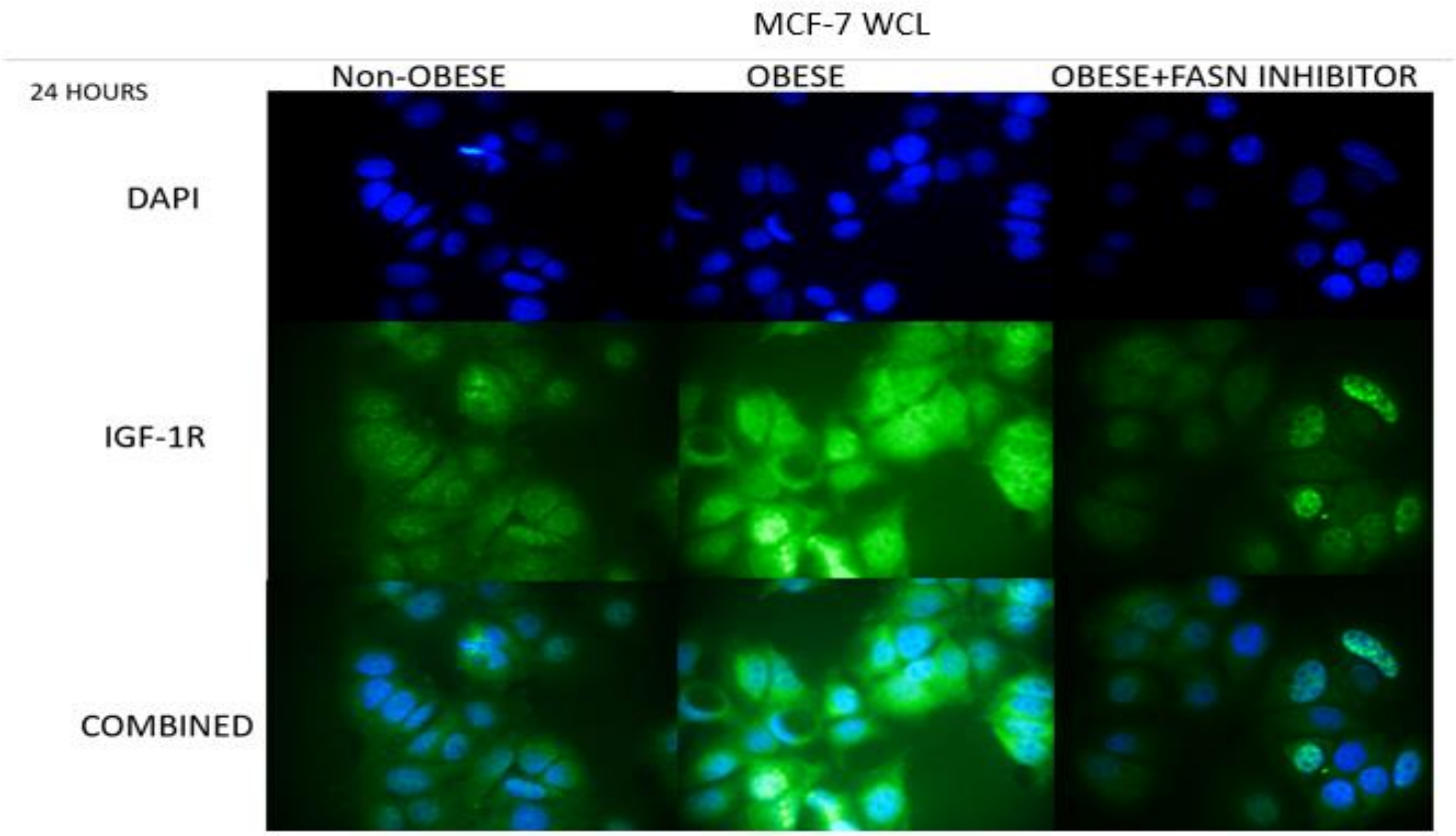

Figure 5 A.

To provide a visualization of the IGF-1 receptor in response to FASN inhibition, MCF-7 Cells were treated with 2% non-obese sera, 2% obese sera, and 2% obese sera with a FASN inhibitor (TVB-3166) for 24 hours. The treated cells were probed for IGF-1β (green) followed by the secondary FITC (red) conjugated antibody. The nuclei were stained using DAPI nuclear stain (blue). The images were obtained using confocal microscopy.

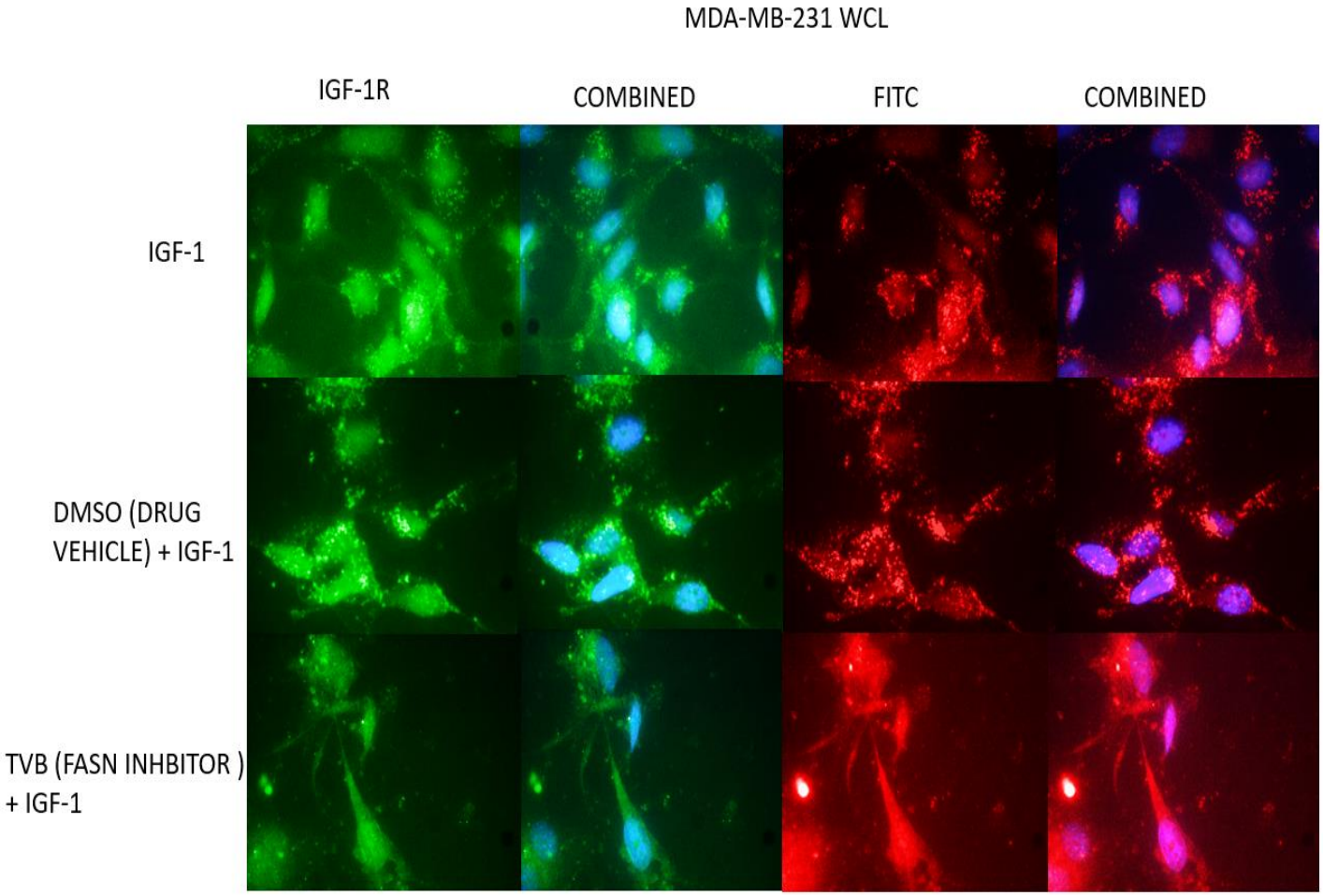

Figure 5 B

To visualize the localization of the IGF-1 receptor, MDA-MB-231 cells were pre-treated with or without 200nM of TVB-3166 or DMSO for 72 hours and exposed to IGF-1. From top to bottom cells were treated with either IGF-1, DMSO + IGF-1, or TVB-3166 +I*GF-1.* IGF-1R was probed for using anti-IGF-1Rβ antibody (green) followed by secondary FITC (red) conjugated antibody. The nuclei were stained using DAPI nuclear stain (blue). The images were visualized using confocal microscopy.

Chapter 3: Concluding Remarks and Future Directions

3.1 Conclusion

In summary, this study provided mechanistic insight as to how obesity contributes to a more aggressive breast cancer. The IGF-1R receptor is correlated with breast cancer drug resistance as well as aggressiveness, thus its increased localization in response to obese sera exposure highlights a potential therapeutic target for treating obese individuals with breast cancer. Also, we previously recorded an increase in FASN expression when breast cancer cells were exposed to obese sera vs non-obese. The use of a FASN inhibitor, TVB-3166, abrogated both the membrane localization and phosphorylation of IGF-1R. Moreover, this study found a FASN dependent mechanism of breast cancer progression through the localization and activation of the IGF-1R in response to obese conditions. Through this study, we have provided both reverse translational insight for a mechanism of a drug currently entering clinical trials and a potential obesity-targeted therapy for breast cancer patients.

3.2 Future Directions

There are two main questions I would like to answer for future directions from this study. Namely, how is the IGF-1R being localized to the membrane in response to FASN and obese sera exposure and how is FASN upregulated in obesity? The connection to an obese environment and metabolic programming for lipogenesis, such as increasing FASN expression is a popular area of research not only for cancer but also for non-alcoholic fatty liver disease. The mechanisms are still being elucidated as to how FASN is transcriptionally controlled. Investigating this mechanism would hold significant clinical relevance due to the growing number of cancer therapeutics targeting cancer metabolism and in particular its ability to transition to a lipogenic phenotype via upregulating FASN expression. Obesity is associated with both an increase in insulin and IGF-1, which both contribute to the activation of Akt and mTORC1. I would like to investigate the role of Akt and mTORC1 in lipogenic programming through FASN in obesity-induced breast cancer. Studies have shown mTORC1 to stabilize FASN mRNA leading to and enhance gene expression of FASN (67). Also, Akt seems to play a pivotal role of FASN expression through the SREBP family of transcription factors (68, 69). A few studies are highlighting the importance of membrane protein localization in response to palmitoylation. Granted the IGF-1R does not have any known residues to be palmitoylated, there are many membrane proteins with known residues that are palmitoylated that are essential for RTK localization and signaling. Flotillin-1 requires palmitoylation for function as is associated with IGF-1R membrane localization, however, the model has not been tested in breast cancer (54). Moreover, I would like to transition into the roles of the flotillin proteins and cancer aggression. There have been studies illustrating the effects of flotillins in breast cancer invasiveness and the formation

of invadopodia. The connection between FASN and flotillin proteins is not quite clear and still needs further elucidation. However, the contribution of flotillins to breast cancer progression is established and is associated with aggressiveness through EMT transcription factors such as snail as well as matrix metalloproteases that are responsible for the degradation of the extracellular matrix (70-72). Finally, I would like to expand on current studies showing FASN as a contributor to drug resistance (53, 73). The studies are mainly involved non-small cell lung carcinoma, however, I believe the mechanisms could translate over to breast cancer. In summary, my future directions involve the investigation of FASN in breast cancer as well as its programming and its downstream effects such as invasion and metastasis.

References

1. Bombonati A, Sgroi DC. The molecular pathology of breast cancer progression. The Journal of Pathology. 2011;223(2):308-18.
2. Desantis CE, Ma J, Gaudet MM, Newman LA, Miller KD, Goding Sauer A, et al. Breast cancer statistics, 2019. CA: A Cancer Journal for Clinicians. 2019;69(6):438-51.
3. Ih A. A Review on Aetio-Pathogenesis of Breast Cancer. Journal of Genetic Syndromes & Gene Therapy. 2013;04(05).
4. Yates LR, Gerstung M, Knappskog S, Desmedt C, Gundem G, Van Loo P, et al. Subclonal diversification of primary breast cancer revealed by multiregion sequencing. Nature Medicine. 2015;21(7):751-9.
5. Engmann NJ, Golmakani MK, Miglioretti DL, Sprague BL, Kerlikowske K. Population-Attributable Risk Proportion of Clinical Risk Factors for Breast Cancer. JAMA Oncology. 2017;3(9):1228.
6. Polyak K. Breast cancer: origins and evolution. Journal of Clinical Investigation. 2007;117(11):3155-63.
7. Van Keymeulen A, Rocha AS, Ousset M, Beck B, Bouvencourt G, Rock J, et al. Distinct stem cells contribute to mammary gland development and maintenance. Nature. 2011;479(7372):189-93.
8. Rakha EA, Green AR. Molecular classification of breast cancer: what the pathologist needs to know. Pathology. 2017;49(2):111-9.
9. Pasculli B, Barbano R, Parrella P. Epigenetics of breast cancer: Biology and clinical implication in the era of precision medicine. Seminars in Cancer Biology. 2018;51:22-35.
10. Giro-Perafita A, Palomeras S, Lum DH, Blancafort A, Vinas G, Oliveras G, et al. Preclinical Evaluation of Fatty Acid Synthase and EGFR Inhibition in Triple-Negative Breast Cancer. 2016;22(18):4687-97.
11. Picon-Ruiz M, Morata-Tarifa C, Valle-Goffin JJ, Friedman ER, Slingerland JM. Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention. CA: A Cancer Journal for Clinicians. 2017;67(5):378-97.
12. Balaban S, Shearer RF, Lee LS, Van Geldermalsen M, Schreuder M, Shtein HC, et al. Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty acids drive breast cancer cell proliferation and migration. Cancer & Metabolism. 2017;5(1).
13. Lionetti L, Mollica MP, Lombardi A, Cavaliere G, Gifuni G, Barletta A. From chronic o vernutrition to insulin resistance: The role of fat-storing capacity and inflammation. Nutrition, Metabolism and Cardiovascular Diseases. 2009;19(2):146-52.
14. Lee B-C, Kim M-S, Pae M, Yamamoto Y, Eberlé D, Shimada T, et al. Adipose Natural Killer Cells Regulate Adipose Tissue Macrophages to Promote Insulin Resistance in Obesity. Cell Metabolism. 2016;23(4):685-98.
15. Schmidt S, Monk JM, Robinson LE, Mourtzakis M. The integrative role of leptin, oestrogen and the insulin family in obesity-associated breast cancer: potential effects of exercise. Obesity Reviews. 2015;16(6):473-87.
16. Bowers LW, Rossi EL, Oâ€™Flanagan CH, Degraffenried LA, Hursting SD. The Role of the Insulin/IGF System in Cancer: Lessons Learned from Clinical Trials and the Energy Balance-Cancer Link. Frontiers in Endocrinology. 2015;6.
17. Bowers LW, Brenner AJ, Hursting SD, Tekmal RR, Degraffenried LA. Obesity-associated systemic interleukin-6 promotes pre-adipocyte aromatase expression via increased breast cancer cell prostaglandin E2 production. 2015;149(1):49-57.

18. Bowers LW, Cavazos DA, Maximo IX, Brenner AJ, Hursting SD, deGraffenried LA. Obesity enhances nongenomic estrogen receptor crosstalk with the PI3K/Akt and MAPK pathways to promote in vitro measures of breast cancer progression. Breast Cancer Research. 2013;15(4):R59.
19. Hursting SD, Lavigne JA, Berrigan D, Perkins SN, Barrett JC. Calorie Restriction, Aging, and Cancer Prevention: Mechanisms of Action and Applicability to Humans. Annual Review of Medicine. 2003;54(1):131-52.
20. Castoldi A, Naffah De Souza C, Câmara NOS, Moraes-Vieira PM. The Macrophage Switch in Obesity Development. Frontiers in Immunology. 2016;6.
21. Petersen MC, Shulman GI. Mechanisms of Insulin Action and Insulin Resistance. Physiological Reviews. 2018;98(4):2133-223.
22. Alipourfard I, Datukishvili N, Mikeladze D. TNF-α Downregulation Modifies Insulin Receptor Substrate 1 (IRS-1) in Metabolic Signaling of Diabetic Insulin-Resistant Hepatocytes. Mediators of Inflammation. 2019;2019:1-6.
23. Haywood NJ, Slater TA, Matthews CJ, Wheatcroft SB. The insulin like growth factor and binding protein family: Novel therapeutic targets in obesity & diabetes. Molecular Metabolism. 2018.
24. Poloz Y, Stambolic V. Obesity and cancer, a case for insulin signaling. Cell Death & Disease. 2015;6(12):e2037-e.
25. Liu Y, Wu M, Ling J, Cai L, Zhang D, Gu HF, et al. Serum IGFBP7 levels associate with insulin resistance and the risk of metabolic syndrome in a Chinese population. 2015;5:10227.
26. Girnita L, Worrall C, Takahashi S-I, Seregard S, Girnita A. Something old, something new and something borrowed: emerging paradigm of insulin-like growth factor type 1 receptor (IGF-1R) signaling regulation. Cellular and molecular life sciences. 2014;71(13):2403-27.
27. Lemmon MA, Schlessinger J. Cell Signaling by Receptor Tyrosine Kinases. Cell. 2010;141(7):1117-34.
28. Heskamp S, Boerman OC, Molkenboer-Kuenen JDM, Wauters CA, Strobbe LJA, Mandigers CMPW, et al. Upregulation of IGF-1R Expression during Neoadjuvant Therapy Predicts Poor Outcome in Breast Cancer Patients. 2015;10(2):e0117745.
29. Engdahl TB, Frost FG, Schech AJ, Elliott SL. Viability and migratory effects of IGF-1R inhibitor, PQ401, on triple negative breast cancer cells. Bios. 2019;90(1):14-9.
30. Björner S, Rosendahl AH, Simonsson M, Markkula A, Jirström K, Borgquist S, et al. Combined and individual tumor-specific expression of insulin-like growth factor-I receptor, insulin receptor and phospho-insulin-like growth factor-I receptor/insulin receptor in primary breast cancer: Implications for prognosis in different treatment gro. Oncotarget. 2017;8(6).
31. Courtney KD, Corcoran RB, Engelman JA. The PI3K Pathway As Drug Target in Human Cancer. Journal of Clinical Oncology. 2010;28(6):1075-83.
32. Fraser J, Ainara, Simpson J, Gammoh N. Interplay of autophagy, receptor tyrosine kinase signalling and endocytic trafficking. Essays In Biochemistry. 2017;61(6):597-607.
33. Caron A, Richard D, Laplante M. The Roles of mTOR Complexes in Lipid Metabolism. Annual Review of Nutrition. 2015;35(1):321-48.
34. Chirala SS, Wakil SJ. Structure and function of animal fatty acid synthase. 2004;39(11):1045-53.

35. Buckley D, Duke G, Heuer TS, O'Farrell M, Wagman AS, McCulloch W, et al. Fatty acid synthase – Modern tumor cell biology insights into a classical oncology target. Pharmacology & Therapeutics. 2017;177:23-31.
36. Chirala SS, Jayakumar A, Gu ZW, Wakil SJ. Human fatty acid synthase: Role of interdomain in the formation of catalytically active synthase dimer. 2001;98(6):3104-8.
37. Menendez JA, Lupu R. Fatty acid synthase and the lipogenic phenotype in cancer pathogenesis. Nat Rev Cancer. 2007;7(10):763-77.
38. Kohnz RA, Mulvihill MM, Chang JW, Hsu K-L, Sorrentino A, Cravatt BF, et al. Activity-Based Protein Profiling of Oncogene-Driven Changes in Metabolism Reveals Broad Dysregulation of PAFAH1B2 and 1B3 in Cancer. 2015;10(7):1624-30.
39. Jones SF, Infante JR. Molecular pathways: fatty acid synthase. Clinical Cancer Research. 2015;21(24):5434-8.
40. Lupu R, Menendez JA. Targeting fatty acid synthase in breast and endometrial cancer: An alternative to selective estrogen receptor modulators? Endocrinology. 2006;147(9):4056-66.
41. Benjamin DI, Li DS, Lowe W, Heuer T, Kemble G, Nomura DK. Diacylglycerol metabolism and signaling is a driving force underlying FASN inhibitor sensitivity in cancer cells. ACS chemical biology. 2015;10(7):1616-23.
42. Bandyopadhyay S, Zhan R, Wang Y, Pai SK, Hirota S, Hosobe S, et al. Mechanism of Apoptosis Induced by the Inhibition of Fatty Acid Synthase in Breast Cancer Cells. Cancer Research. 2006;66(11):5934.
43. Cui Y, Xing P, Wang Y, Liu M, Qiu L, Ying G, et al. NADPH accumulation is responsible for apoptosis in breast cancer cells induced by fatty acid synthase inhibition. Oncotarget. 2017;8(20):32576.
44. Patel M, Infante J, Von Hoff D, Jones S, Burris H, Brenner A, et al. Abstract CT203: Report of a first-in-human study of the first-in-class fatty acid synthase (FASN) inhibitor TVB-2640. Cancer Research. 2015;75(15 Supplement):CT203-CT.
45. Falchook G, Patel M, Infante J, Arkenau H-T, Dean E, Brenner A, et al. Abstract CT153: First in human study of the first-in-class fatty acid synthase (FASN) inhibitor TVB-2640. AACR; 2017.
46. Brenner A, Falchook G, Patel M, Infante J, Arkenau H, Dean E, et al. Abstract P6-11-09: Heavily pre-treated breast cancer patients show promising responses in the first in human study of the first-In-class fatty acid synthase (FASN) inhibitor, TVB-2640 in combination with paclitaxel. AACR; 2017.
47. Alwarawrah Y, Hughes P, Loiselle D, Carlson DA, Darr DB, Jordan JL, et al. Fasnall, a selective FASN inhibitor, shows potent anti-tumor activity in the MMTV-Neu model of HER2+ breast cancer. Cell chemical biology. 2016;23(6):678-88.
48. Corominas-Faja B, Vellon L, Cuyàs E, Buxó M, Martin-Castillo B, Serra D, et al. Clinical and therapeutic relevance of the metabolic oncogene fatty acid synthase in HER2+ breast cancer. Histology and histopathology. 2017;32(7):687.
49. Menendez JA, Lupu R. Fatty acid synthase regulates estrogen receptor-α signaling in b reast cancer cells. Oncogenesis. 2017;6(2):e299-e.
50. Coleman DT, Gray AL, Kridel SJ, Cardelli JA. Palmitoylation regulates the intracellular trafficking and stability of c-Met. Oncotarget. 2016;7(22):32664-77.

51. Ernst AM, Syed SA, Zaki O, Bottanelli F, Zheng H, Hacke M, et al. S-Palmitoylation Sorts Membrane Cargo for Anterograde Transport in the Golgi. Developmental Cell. 2018;47(4):479-93.e7.
52. Kristin, Kharbanda A, Stypulkowski E, Cao X-J, Wang W, Benjamin, et al. Inhibition of DHHC20-Mediated EGFR Palmitoylation Creates a Dependence on EGFR Signaling. 2016;62(3):385-96.
53. Ali A, Levantini E, Teo JT, Goggi J, Clohessy JG, Wu CS, et al. Fatty acid synthase mediates EGFR palmitoylation in EGFR mutated non-small cell lung cancer. EMBO Molecular Medicine. 2018;10(3):e8313.
54. Jang D, Kwon H, Jeong K, Lee J, Pak Y. Essential role of flotillin-1 palmitoylation in the intracellular localization and signaling function of IGF-1 receptor. J Cell Sci. 2015;128(11):2179-90.
55. Wu J, Du J, Fu X, Liu B, Cao H, Li T, et al. Icaritin, a novel FASN inhibitor, exerts anti-melanoma activities through IGF-1R/STAT3 signaling. Oncotarget. 2016;7(32):51251.
56. Catenacci VA, Hill JO, Wyatt HR. The obesity epidemic. Clin Chest Med. 2009;30(3):415-44, vii.
57. Neuhouser ML, Aragaki AK, Prentice RL, Manson JE, Chlebowski R, Carty CL, et al. Overweight, obesity, and postmenopausal invasive breast cancer risk: a secondary analysis of the women's health initiative randomized clinical trials. JAMA oncology. 2015;1(5):611-21.
58. Hormones E, Group BCC. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. Journal of the National Cancer Institute. 2002;94(8):606-16.
59. Drew BG, Hamidi H, Zhou Z, Villanueva CJ, Krum SA, Calkin AC, et al. Estrogen receptor (ER) α-regulated lipocalin 2 expression in adipose tissue links obesity with breast cancer progression. Journal of Biological Chemistry. 2015;290(9):5566-81.
60. Zemel MB. Regulation of adiposity and obesity risk by dietary calcium: mechanisms and implications. Journal of the American College of Nutrition. 2002;21(2):146S-51S.
61. Kohnz RA, Mulvihill MM, Chang JW, Hsu KL, Sorrentino A, Cravatt BF, et al. Activity-Based Protein Profiling of Oncogene-Driven Changes in Metabolism Reveals Broad Dysregulation of PAFAH1B2 and 1B3 in Cancer. ACS Chem Biol. 2015;10(7):1624-30.
62. Louie SM, Roberts LS, Nomura DK. Mechanisms linking obesity and cancer. Biochimica Et Biophysica Acta (bba)-Molecular And Cell Biology Of Lipids. 2013;1831(10):1499-508.
63. Baron A, Migita T, Tang D, Loda M. Fatty acid synthase: a metabolic oncogene in prostate cancer? Journal of cellular biochemistry. 2004;91(1):47-53.
64. Crunkhorn S. Breast cancer: FASN inhibitor increases survival. Nature Reviews Drug Discovery. 2016;15(8):532.
65. Wang Z, Liu G, Mao J, Xie M, Zhao M, Guo X, et al. IGF-1R Inhibition Suppresses Cell Proliferation and Increases Radiosensitivity in Nasopharyngeal Carcinoma Cells. Mediators of inflammation. 2019;2019.
66. Ventura R, Mordec K, Waszczuk J, Wang Z, Lai J, Fridlib M, et al. Inhibition of de novo Palmitate Synthesis by Fatty Acid Synthase Induces Apoptosis in Tumor Cells by Remodeling Cell Membranes, Inhibiting Signaling Pathways, and Reprogramming Gene Expression. EBioMedicine. 2015;2(8):808-24.

67. Lee G, Zheng Y, Cho S, Jang C, England C, Dempsey JM, et al. Post-transcriptional Regulation of De Novo Lipogenesis by mTORC1-S6K1-SRPK2 Signaling. Cell. 2017;171(7):1545-58.e18.
68. Laplante M, Sabatini DM. mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis: Fig. 1. Proceedings of the National Academy of Sciences. 2010;107(8):3281-2.
69. Jessica, Hui, Menon S, Liu S, Yecies D, Alex, et al. Akt Stimulates Hepatic SREBP1c and Lipogenesis through Parallel mTORC1-Dependent and Independent Pathways. Cell Metabolism. 2011;14(1):21-32.
70. Jang D, Kwon H, Choi M, Lee J, Pak Y. Sumoylation of Flotillin-1 promotes EMT in metastatic prostate cancer by suppressing Snail degradation. Oncogene. 2019.
71. Planchon D, Morris ER, Genest M, Comunale F, Vacher S, Bièche I, et al. MT1-MMP targeting to endolysosomes is mediated by upregulation of flotillins. J Cell Sci. 2018;131(17):jcs218925.
72. Upregulation of flotillin-1 promotes invasion and metastasis by activating TGF-β signaling in nasopharyngeal carcinoma. 2016.
73. Cao S, Cui Y, Xiao H, Mai M, Wang C, Xie S, et al. Upregulation of flotillin-1 promotes invasion and metastasis by activating TGF-β signaling in nasopharyngeal carcinoma. Oncotarget. 2016;7(4):4252.